New Roles of Plant ARGONAUTE Proteins

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ARGONAUTE (AGO) proteins are a conserved family of eukaryotic proteins that act as core components of RNA silencing by loading small RNAs (sRNAs) derived from plants and other interacting organisms. sRNAs associate with AGOs to form RNA-induced silencing complexes (RISCs), which target complementary RNA or DNA sequences to repress gene expression by post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS), respectively.

ARGONAUTE (AGO) protein small RNAs (sRNAs) loading gene silencing functions

1. Introduction

Phylogenetic analyses have revealed three major clades of angiosperm AGO proteins, which are named after *Arabidopsis* AGOs: AGO1/5/10, AGO2/3/7, and AGO4/6/8/9 [1]. Monocots have also evolved a specific AGO, AGO18, which forms its own subclade closest to the AGO1/5/10 subclade [1]. AGO17, whose evolutionary position has fluctuated outside and inside the AGO1 clade [2][3][4][5], was first identified as a rice-specific AGO family member [3] and was subsequently discovered in several other Poaceae species [6]. A recent phylogenetic tree based on genomic and transcriptomic data from more plant species revealed additional divergence of eudicot AGO10 into AGO10a and AGO10b [5]. Notably, another AGO-like subclade exists in early land plants, such as hornworts, lycophytes, ferns, and gymnosperms, but has been lost specifically in angiosperms [7].

Traditional functions of AGOs are inseparable from sRNA loading, and their multifaceted roles in plant development and environmental response are determined by the types of sRNAs with which they associate. Intensive studies of miRNA–AGO loading have revealed the mechanisms by which sRNAs are sorted into various AGOs, depending on the 5′ terminal nucleotide, the sRNA length, and the sequence and structural features of the sRNA duplex and sRNA precursors [8][9][10][11]. The spatiotemporal expression pattern of the AGO and its sRNA partner is another major factor that affects sRNA sorting [12]. However, sRNA loading does not only initiate target gene silencing through an sRNA-mediated mechanism; it has broader functions in balancing distinct classes of sRNAs, modulating plant development and defense by cross-regulation within the AGO protein family, and enabling intra- and extracellular sRNA movement.

2. Nontraditional Functions of AGO Proteins

Although the biological functions of AGOs have been intensively studied in past decades, because of the diversity of this large family and the independent expansion of each subclade after species differentiation [7], the overall functions of AGO proteins are still underestimated. AGO8 was long thought to be a pseudogene in *Arabidopsis* because of a predicted splice-inducing frame shift in its putative coding sequence, which suggested it would form a truncated and nonfunctional protein [13]. However, cytological analysis revealed a more frequent occurrence of mutant ovules in two independent *ago8* mutant alleles, and other mutants in the AGO4 clade, including *ago4*, *ago6*, and *ago9*, showed a similar phenotype. Quantitative real-time PCR revealed upregulation of *AGO8* in ovules of *ago4 ago9*, implying that AGO8 might play a compensatory role during female gametophyte development [14]. Assays with fluorescent-protein-tagged AGO8 revealed that it was specifically expressed in egg cells of *Arabidopsis* and was localized in the cytoplasm [15]. Although AGOs in the same clade typically show similar functions, AGO3 from the AGO2/3/7 clade (the last characterized AGO member in *Arabidopsis*) is an exception. The activity of AGO3 is similar to that of AGO4, and it binds 24 nt siRNAs that overlap with those of AGO4, but it differs in function from its closest paralog, AGO2, which functions mainly in antiviral defense [16]. Unexpected functions of AGO8 in tobacco defense against herbivory have also been revealed. AGO8-silenced transgenic *Nicotiana attenuata* are hyper-susceptible to herbivore attack, owing to reduced biosynthesis of defense metabolites. Although the differential expression of miRNAs and target genes in response to AGO8 silencing as well as the protein structural basis for sRNA loading into AGO8 were analyzed, evidence for direct AGO8 protein–sRNA interaction remains to be generated [17].

The nuclear roles of AGO proteins, including roles in DNA repair and transcription, have recently been summarized in a review by Bajczyk [18]. Of particular interest are new findings suggesting that AGO proteins may play transcription-factor-like roles. Several plant AGO proteins can directly bind to chromatin to regulate gene expression. A chromatin immunoprecipitation (ChIP) assay of AGO1 showed a chromatin occupancy pattern analogous to that of RNA polymerase II (Pol II), suggesting that AGO1 can promote gene transcription, especially in response to plant hormones and stresses [19]. To date, the molecular mechanisms by which AGO1 helps to regulate the transcription of nuclear genes remain unclear. The previous report proposed that the chromatin remodeling factor SWI2/SNF2 and DCL1-dependent sRNAs, which are probably not miRNAs, may participate in this process [19]. However, a later study found that the ATPase subunit of SWI2/SNF2 was involved in the production of miRNAs [20]. Generation of the sRNAs required for AGO1 chromatin binding appeared to be dependent on the miRNA biogenesis machinery [19]. Thus, impaired chromatin binding of AGO1 in mutants of SWI2/SNF2 complex subunits may be due to misregulation of sRNA biogenesis. A later study in rice revealed a new mechanism of transcriptional modulation mediated by AGO2 [21]. OsAGO2 binds directly to the promoter of *OsHXK1* to regulate ROS generation during anther development by a mechanism that involves DNA methylation [21]. In contrast to AGO1, whether the nuclear regulatory process of AGO2 depends on its associated sRNAs is still unknown. AGO1 also participates in a co-transcriptional repression mechanism that determines plant flowering via the floral repressor locus *FLOWERING LOCUS C* (*FLC*) [22]. AGO1 physically associates with co-transcriptional regulators, such as components of Pol II and splicing-related proteins, to regulate antisense RNA *COOLAIR* processing, ultimately creating a local chromatin-silencing environment that determines the output of the sense transcript, *FLC* [22].

Consistent with its involvement in RNA processing, AGO1 also participates in mRNA intron splicing, a function that is conserved in animals [23]. The binding regions of AGO proteins are enriched at intronic regions of mRNA transcripts, a phenomenon consistent with the intron retention observed in *ago* mutants and the proposed function of AGOs in alternative splicing [24]. IP-mass spectra also revealed several novel mRNA-binding interactors of AGOs that facilitate their intron recognition [24]. Nonetheless, the detailed nuclear regulatory mechanism of AGOs requires further investigation. In human cell cultures, AGO2 participates in mRNA quality control by sensing abnormal and nascent peptide chains in the cytosol [25]; whether this mechanism is conserved in plants remains unclear.

Phase separation is a process in which multiple biomolecules assemble autonomously to form membraneless cellular structures; these independent and flexible sub-environments separate the vast diversity of biochemical reactions that occur within the limited space of a single cell. Intracellular liquid–liquid phase separation (LLPS) occurs during ongoing processing of miRNAs in the D-body [26]. Interestingly, although AGO4 does not contain domains involved in LLPS, it often colocalizes with discrete nuclear speckles called Cajal bodies (important functional nuclear foci for RdDM) [27] and AB-bodies [28]. These two distinct nuclear AGO4 bodies were representatives of phase separation and were structurally independent of one another [28]. Several other AGO proteins (AGO1, AGO2, and AGO3) are predicted to harbor prion-like domains [29] that may provide the driving force for LLPS formation [26]. Although experimental confirmation for a role of AGOs in phase separation is necessary, a study on AGO1 subcellular localization during different phases of the cell cycle revealed a complex distribution pattern with foci in the nucleus and cytosol that is consistent with the LLPS potential of AGO1 [30].

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